difference between the two was slight. The average percentage of bone magnesium in Group A rats was 0.714 after 56 days and 0.737 after 74 days. For Group B rats it was 0.676 after 56 days and 0.691 after 74 days, indicating that the content of bone magnesium was not decreasing as restriction to the milk diet continued.

Since, in addition to the normal serum magnesium and absence of deficiency symptoms, the magnesium content of bone did not continually decrease during the experimental period, it is concluded that cow milk contains enough magnesium to prevent magnesium deficiency in rats. This suggests that the magnesium requirement of rats is either (a) lower than that of calves, or (b) rats are better able than calves to utilize the magnesium of cow milk.

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Anticatalase Activity of Sulfanilamide and Related Compounds. III. Oxygen Tension and Bacteriostasis in Pneumococcal Cultures.

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In previous publications[2-5] the authors have investigated the theory, originally proposed by Locke,[1] that the retardation of growth of certain microörganisms in the presence of sulfanilamide may be primarily the result of the accumulation of hydrogen peroxide. This accumulation is presumed to arise through the inhibition of catalase by sulfanilamide which has been activated by oxidation. It was demonstrated[2] that sulfanilamide and many structurally related compounds have an appreciable anticatalase activity which is frequently enhanced by oxidative processes such as those involved in ultraviolet irradiation and[5] that bacteriostasis of Type I pneumococcus *in vitro* is accompanied by a correspondingly marked accumulation of hydrogen peroxide in the culture.

The formation of hydrogen peroxide requires time and oxygen,

1 Locke, A., Main, E. R., and Mellon, R. R., *Science*, 1938, **88**, 620.

2 Main, E. R., Shinn, L. E., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 272.

3 Locke, A., Main, E. R., and Mellon, R. R., *J. Immunol.*, 1939, **36**, 183.

4 Mellon, R. R., *Modern Hospital*, 1938, **51**, 53.

5 Shinn, L. E., Main, E. R., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 591.

"points of sufficient importance as possible limiting factors in the application of sulfanilamide therapy to occluded and rapidly developing infection to warrant inquiry and test."[3] The dependence of the inhibitory power of sulfanilamide on oxygen has been examined experimentally *in vitro* with reference to the Type I pneumococcus and the results are herewith reported.

The cultures were grown in 50 cc Erlenmeyer flasks each containing 10 cc of veal-heart-infusion-broth, autoclaved immediately before use. Glucose (0.4%) and blood (5×10^{-5} cc) were then added to each flask. Sulfanilamide (10 mg %) was added where required. All flasks were seeded with 0.025-0.1 cc of a 6-hour broth culture of Type I pneumococcus, the exact amount for a given experiment being determined by the degree of growth of the seeding culture. Reduced oxygen tension was obtained by placing the flasks in vacuum jars and evacuating and refilling with nitrogen to atmospheric pressure until the required concentration of oxygen was theoretically present. In the last jar of each series an oxygen absorbent, sodium pyrogallate or hydrosulfite, was used to remove further traces of oxygen. All cultures were incubated 16-17 hours, at the end of which time growth was determined by turbidimetric comparisons. Plate-counts were avoided because of the chaining which occurs in the presence of sulfanilamide.

The results, given in Table I, show that decrease in oxygen tension is accompanied by decrease in the inhibition of growth caused by sulfanilamide. The average inhibition in the presence of air (21% oxygen) is 54%. At 10% oxygen the inhibition is 40% and the decrease continues until a concentration of 1% of oxygen is reached, where the inhibition is essentially zero. When the concentration is reduced to 0.04% there is uniformly a stimulation of growth in the presence of sulfanilamide. On still further reduction of the available oxygen by the presence of pyrogallate or hydrosulfite, the inhibition resulting from sulfanilamide reappears.

No hydrogen peroxide was detected (limit about 6 γ/cc) in the cultures which contained no sulfanilamide.* Peroxide was present in concentrations of 10-25 γ/cc in the sulfanilamide-containing cultures grown in 21 or 10% oxygen. No peroxide was detected in any cultures at the lower oxygen levels. This result accords well with the abrupt drop in inhibition (average) between the 10 and 5% oxygen levels.

It is difficult to explain the inhibition at low oxygen tension as a

* This would be anticipated from the constitution and relative old age of the cultures.

TABLE I.

Effect of Concentration of Oxygen in Gaseous Environment on Inhibition of Growth of Type I Pneumococcus by Sulfanilamide.

% Oxygen Present	Exp. 1			Exp. 2			Exp. 3			Exp. 4			Exp. 5			Avg I
	Gc	Gs	I	Gc	Gs	I	Gc	Gs	I	Gc	Gs	I	Gc	Gs	I	1-5
21	92	12	+87	102	31	+69	124	104	+16	72	21	+71	140	102	+27	+54
10	120	21	+82	142	102	+14	164	124	+24	140	86	+39	180	102	+43	+40
5	110	102	+ 7	142	102	+14	164	144	+12	102	102	0	120	102	+15	+10
2.5	102	102	0	112	112	0	164	144	+12	102	86	+16	140	120	+14	+ 8
1.0	102	102	0	122	92	+25	144	144	0	86	86	0	102	120	—18	+ 1
0.04	81	110	—25	62	92	—49	84	116	—36	76	86	—13	110	130	—18	—28
<0.04*	92	27	+71	72	32	+55	76	64	+16	62	13	+79	92	72	+22	+49

Gc—Growth in control culture.

Gs—Growth in culture containing 10 mg% of sulfanilamide.

$$\text{I—\% inhibition} = \frac{Gc - Gs}{Gc} \times 100.$$

*Oxygen tension reduced to 0.04% and an oxygen absorbent added to remove the remainder.

The growth unit employed is an arbitrary value based on turbidity-readings.

result of peroxide accumulation. As the oxygen tension reaches a sufficiently low level another mechanism may appear. While the anticatalase activity of sulfanilamide apparently depends upon activation of the *p*-amino group,[2] the sulfonamido group may become reactive under anaërobic conditions. Reduction to sulfides, known to be highly toxic, may account for the reappearance of inhibition under extreme anaërobic conditions (*cf.*, last line of Table I). The great volume of clinical and experimental work carried out with the sulfonamide compounds has shown that the presence of the sulfur group is essential to their effectiveness. Increased growth in the presence of sulfanilamide in the next to lowest oxygen concentration could be explained as stimulation by very small quantities of substances which are toxic in larger amounts. This condition of extreme anaërobiosis would probably not obtain clinically in pneumococcal or streptococcal infections.

Little attention has been given to the bacteriostasis of anaërobes or infections with anaërobes insofar as sulfanilamide is concerned. However, Spray[6] has shown that certain spore-forming anaërobes are susceptible to bacteriostasis by this compound. It may be that in such cases the anaërobic type of inhibition which has been demonstrated here will prove pertinent.

A significant comparison can be made between the concentration of oxygen required for adequate bacteriostasis and the concentration available *in vivo*. The oxygen present in the plasma of normal arterial blood is stated to be 0.6%.[7] Assuming the solubility of oxygen in broth to be essentially the same as in water and assuming that at the higher levels of oxygen concentration equilibrium is attained, the oxygen present in the cultures with atmospheres containing 21, 10, and 5% oxygen would be respectively 0.5, 0.24, and 0.12%. Thus under normal conditions the amount of oxygen present in plasma is adequate for support of a high degree of inhibition. The dependence of sulfanilamide upon oxygen indicates the advisability of maintaining or increasing, where possible, the oxygen supply at the lesion.

While this material was being prepared for publication there appeared 2 excellent articles which constitute essential confirmation, with respect to the streptococcus, of the mechanism which we have proposed. Fox, German, and Janeway[8] clearly demonstrated by

[6] Spray, R. S., *J. Lab. and Clin. Med.*, 1938, **23**, 609.

[7] Sollmann, T., *Manual of Pharmacology*, W. B. Saunders Co., Philadelphia, 1936, page 753.

[8] Fox, C. L., German, B., and Janeway, C. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 184.

potentiometric methods that cultures of the hemolytic streptococcus undergoing inhibition by sulfanilamide maintain an elevated potential indicating the presence of oxidizing substances. The normal growth in cultures without sulfanilamide was accompanied by a fall in potential. A similar fall in potential occurred in sulfanilamide-containing cultures after inhibition was overcome. The presence of cysteine or reduced access to oxygen diminished or obliterated the inhibitory power of sulfanilamide. The addition of catalase decreased the potentials attained and the degree of stasis. This effect of added catalase implies that the substance responsible for elevated potential and bacteriostasis is hydrogen peroxide. Fox and his coworkers draw no conclusion regarding the nature of the oxidizing substance. In view of the method employed, which is an extremely delicate but non-specific test for oxidizing and reducing agents, their caution is fully justified.

Warren, Street, and Stokinger[9] presented at the same time essentially similar results by the same methods. They have extended the study by testing some related compounds and showing that, among others, the presence of *p*-acetyl sulfanilamide, considered by most workers to be a completely inactive compound, caused no rise in potential and no inhibition.† Sulfapyridine produced inhibition and caused an elevated potential. In work soon to be published we have been able to show that *p*-acetyl sulfanilamide produces no inhibition and no peroxide accumulation in the pneumococcus and that sulfapyridine produces inhibition and peroxide accumulation in excess of that produced by sulfanilamide.

Warren, *et al.*, place a somewhat different interpretation on their results than do Fox and his coworkers. The divergence appears to be based largely on differing results with regard to the potential produced by suufanilamide in sterile broth. Variable findings on this point are not surprising in view of the fact that sulfanilamide could, in theory, cause such a rise under proper conditions. Traces of intermediate oxidation products of the *p*-amino group would probably confer an elevated potential. That these may exist in sulfanilamide solutions is indicated by the appreciable anticatalase activity of the non-irradiated compound.[2]

Summary. 1. Reduction of the percentage of oxygen in the

[9] Warren, J., Street, J. A., and Stokinger, H., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 208.

† In [2] it was shown that *p*-acetyl sulfanilamide is distinguished by possessing no anticatalase activity before or after irradiation.

superambient air of broth cultures of the Type I pneumococcus greatly reduced or prevented bacteriostasis by sulfanilamide.

2. When the oxygen concentration was reduced to 0.04% an actual stimulus of growth by sulfanilamide was found.

3. When the oxygen was further reduced by the presence of pyrogallate or hydrosulfite the inhibition by sulfanilamide reappeared.

4. Hydrogen peroxide was detected only in cultures in equilibrium with atmospheres containing 10% oxygen or more. These concentrations correspond to those permitting effective bacteriostasis and are comparable to those obtainable in the plasma.

5. The lack of inhibition at intermediate concentrations shows that oxygen plays a vital rôle in the action of sulfanilamide. The failure to form peroxide at these same concentrations is taken as evidence that oxygen exerts its influence through the agency of hydrogen peroxide. The stimulus and recurring inhibition at the lowest values is interpreted as evidence of the formation of a toxic reduction compound, possibly a sulfide. The latter type of inhibibition may play a rôle in any bacteriostatic effect against anaërobes.

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Advances in the Serological Typing of *Streptococcus hemolyticus.*

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Griffith's[1] technic for typing Group A hemolytic streptococcus has been applied in this laboratory to the classification of more than one thousand strains recovered from a variety of infections. It was possible to type 70% of the organisms examined in 1935 and 1936 by the procedure described previously.[2] At that time several difficulties were pointed out: (1) the elimination of anti-C cross-reactions; (2) the granular character of matt organisms; and (3) the failure of certain strains to be agglutinated by any of the 28 available type-specific sera.

As shown previously[2] interfering cross-reactions due to anti-C substance in the sera could be eliminated by absorbing the sera with

[1] Griffith, F., *J. Hyg.*, 1935, **34**, 542.

[2] Pauli, R. H., and Coburn, A. F., *J. Exp. Med.*, 1937, **65**, 595.